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Molecular Detection of Fungal Communities in the Hawaiian Marine Sponges *Suberites zeteki* and *Mycale armata*▽

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Symbiotic microbes play a variety of fundamental roles in the health and habitat ranges of their hosts. While prokaryotes in marine sponges have been broadly characterized, the diversity of sponge-inhabiting fungi has barely been explored using molecular approaches. Fungi are an important component of many marine and terrestrial ecosystems, and they may be an ecologically significant group in sponge-microbe interactions. This study tested the feasibility of using existing fungal primers for molecular analysis of sponge-associated fungal communities. None of the eight selected primer pairs yielded satisfactory results in fungal rRNA gene or internal transcribed spacer (ITS) clone library constructions. However, 3 of 10 denaturing gradient gel electrophoresis (DGGE) primer sets, which were designed to preferentially amplify fungal rRNA gene or ITS regions from terrestrial environmental samples, were successfully amplified from fungal targets in marine sponges. DGGE analysis indicated that fungal communities differ among different sponge species (*Suberites zeteki* and *Mycale armata*) and also vary between sponges and seawater. Sequence analysis of DGGE bands identified 23 and 21 fungal species from each of the two sponge species *S. zeteki* and *M. armata*, respectively. These species were representatives of 11 taxonomic orders and belonged to the phyla of *Ascomycota* (seven orders) and *Basidiomycota* (four orders). Five of these taxonomic orders (*Malasseziales*, *Corticiales*, *Polyporales*, *Agaricales*, and *Dothideomycetes et Chaetothyriomcetes incertae sedis*) have now been identified for the first time in marine sponges. Seven and six fungal species from *S. zeteki* and *M. armata*, respectively, are potentially new species because of their low sequence identity (≤98%) with their references in GenBank. Phylogenetic analysis indicated sponge-derived sequences were clustered into "marine fungus clades" with those from other marine habitats. This is the first report of molecular analysis of fungal communities in marine sponges, adding depth and dimension to our understanding of sponge-associated microbial communities.

Fungi are ubiquitous and fulfill a wide range of important ecological functions, particularly those associated with nutrient and carbon cycling processes in both marine and terrestrial habitats (17, 59). Yet, our understanding of fungal community diversity and its functioning is still poor compared to our knowledge of bacterial communities. Much of what is known regarding the diversity and functioning of fungi in environments is based on the data derived from culture-based techniques (2, 10, 89). These methods are well known to detect only a small fraction of the fungal community, and therefore, their results provide only a selective, and invariably biased, window on diversity (1, 2, 47, 77). Of an estimated 1.5 million fungal species in existence, only 5 to 10% have been formally described (30–32). In addition, fungi in natural habitats, including those isolated from sponges, in many cases are not able to produce the resting structure, such as spores, and only their vegetative mycelium is available for detailed analysis (23, 38, 57, 64). Hence, identification of these fungi is at best difficult and generally not possible (10). Recently, substantial advances have been made in our knowledge of fungal community ecology through the development and application of molecular techniques that allow direct detection of the fungal taxa from environmental samples. These molecular techniques include, but are not limited to, clone library construction (9, 16), automated rRNA intergenic spacer analysis (67), terminal restriction fragment length polymorphism (20, 50), denaturing gradient gel electrophoresis (DGGE) (3, 4, 56, 76), and temperature gradient gel electrophoresis (21, 75). Despite the wide use of molecular methods to study fungal communities, none of these molecular techniques has been used to explore sponge-associated fungal communities.

Sponges (phylum *Porifera*) contain an estimated 15,000 species in three taxonomic classes: *Calcarea* (calcareous sponges), *Hexactinellida* (glass sponges), and *Demospongiae* (demosponges) (39). Their biomass represents a significant component of benthic communities in the world's oceans (79). As sedentary filter-feeding organisms, sponges are remarkably efficient at obtaining food from the surrounding water and can pump up to 24,000 liters of seawater through a 1-kg sponge per day (33, 62, 69, 83). Any planktonic microbe can become a resident in sponges, provided that it can survive the digestion and immune response in sponges, and can be capable of growing in the microenvironment of the sponge mesohyl (90). In high-microbial-abundance sponges, microbial populations constitute as much as 40% of the sponge tissue volume (81), and microbial densities can reach more than 10^9 cells per ml of sponge tissue (37, 86), several orders of magnitude higher than what is typically found in seawater. Cultivation-dependent and molecular approaches have identified phylogenetically diverse microbial groups, including all three domains of life, i.e., *Bacteria*, *Archaea*, and *Eukarya*, some of which are sponge specific

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TABLE 1. Fungal DGGE-PCR primers (18S or ITS) used to detect unculturable fungi in the Hawaiian marine sponge *Suberites zeteki*

DGGE-PCR set ID	Primers		DGGE-PCR products		Sequenced DGGE-PCR clones		DGGE band no.	Fungal taxon no.	Reference(s)
	Primary PCR[a]	Nested PCR	Size (bp)	Target	No. of fungal inserts	No. of sponge inserts			
1	ITS1/ITS2-GC		350	ITS	0	13	5	0	21
2	ITS1F/ITS2-GC		350	ITS	0	10	7	0	21
7	NS1/GCFung		400	18S	14	0	3	7	56
3	P1-GC/P2		350	ITS1/5.8S	NA[b]	NA	9	NA	25
4	EF4/EF3	EF4/NS3-GC	500	18S	NA	NA	NA	NA	11, 75
5	EF4/fung5	EF4/NS3-GC	500	18S	NA	NA	NA	NA	11, 75
8	NS1/NS8	NS1/NS2 + 10-GC	600	18S	16	1	2	4	43
10	ITS1F/ITS4	ITS3/ITS4-GC	450	ITS	46	15	10	23	4

[a] PCR cycling conditions are described in the cited references, as indicated in the text.
[b] NA, not applicable. Refer to the text for details.

(for reviews, see references 34, 36, 48, 79, and 84). However, previous studies of sponge-associated microbes have been primarily focused on prokaryotes (*Bacteria* and *Archaea*). Eukaryotic microbes, such as dinoflagellates and diatoms, have been reported to be present in marine sponges (7, 14, 15, 72, 74, 87). Detailed studies of eukaryotic microbial communities living within sponges are rare. In particular, fungal communities in marine sponges remain relatively unknown.

In this study, we tested the existing fungal primers in rRNA gene or ITS (internal transcribed spacer) library construction and DGGE analyses to investigate fungal communities associated with the marine sponges *S. zeteki* and *M. armata*, providing the first insight into the unculturable fungal communities in marine sponges.

MATERIALS AND METHODS

Sampling site and sponge collection. Kaneohe Bay is a shallow (less than 20 m), semitropical embayment approximately 13 km long and 4 km broad, located on the northeastern (windward) coast of the island of Oahu, HI. The sponge samples were collected along the shores of Coconut Island, which is situated within Kaneohe Bay and surrounded by 64 acres of coral reef. The island itself covers approximately 29 acres.

Sponge samples were collected in May 2005. Three samples (approximately 6 cm^3) of healthy *S. zeteki* and *M. armata* were collected from each of three individual sponges within a 15-m radius by snorkeling at a depth of 1 to 3 m in Kaneohe Bay, Oahu, HI. They were transferred directly to a Ziploc bag containing seawater from the collection sites, kept on ice, and transported immediately in a cooler back to the laboratory for processing. Sponge tissues for genomic DNA isolation were frozen in liquid nitrogen and then stored at −80°C until use. Samples of seawater were collected in an autoclaved glass bottle (Pyrex bottle) at each collection site and transported back to the laboratory in a cooler. Seawater samples were sequentially filtered through 2- and 0.2-μm cellulose-acetate filters. The filters were frozen in liquid nitrogen until further processing.

Constructing 18S rRNA gene and ITS clone libraries. The total genomic DNA of sponges was extracted from frozen sponge tissues using the FastDNA kit (Qbiogene), according to the manufacturer's instructions. The resulting genomic DNA was used as a PCR template to amplify 18S rRNA gene or the ITS region using ITS1-F/ITS4-B, ITS3/ITS4, NS1/NS8, ITS1F/ITS4, FR1/EF390, ITS1/ITS4, nu-ssu-0817/nu-ssu-1196, and ITS5/ITS4B (59, 88). Cycling conditions were 95°C for 3 min, then 35 cycles at 95°C for 1 min, 48 to 55°C for 1 min, 72°C for 1 min, and a final extension for 10 min at 72°C. PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under UV transillumination. PCR products were purified using the QIAquick PCR purification kit (Qiagen) or QIAquick gel extraction kit (Qiagen) prior to being cloned into the pCR2.1-TOPO vector and being transformed into One Shot Competent *Escherichia coli* cells using the TOPO TA cloning kit (Invitrogen). Positive clones were selected with blue/white screening using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate on LB agar plates supplemented with 100 μg ml^{-1} of ampicillin. The positive clones carrying the correct insert sizes of ITS or 18S rRNA gene fragments were screened using the primers M13f and M13r. The plasmid DNA was purified from 5-ml bacterial cultures grown in LB broth supplemented with 100 μg ml^{-1} of ampicillin using the QIAprep Spin Miniprep kit (Qiagen) and was directly used for sequencing analysis.

DGGE analysis. The total genomic DNA extracted from sponge tissues was used as a PCR template for the amplification of 18S rRNA gene or ITS regions using 11 primer pairs: ITS1/ITS2-GC, ITS1F/ITS2-GC, P1-GC/P2, EF4/EF3, EF4/fung5, EF4/NS3-GC, NS1/GCfung, NS1/NS8, NS1/NS2 + 10-GC, ITS1F/ITS4, and ITS3/ITS4-GC (Table 1). PCR primers ITS1/ITS2-GC, ITS1F/ITS2-GC, P1-GC/P2, and NS1/GCfung were directly used for the amplification of the 18S rRNA gene or ITS fragments for DGGE analysis. The other primer pairs were used in either primary PCR or the nested PCR for DGGE analysis. One microliter of diluted primary PCR product (1:100) was used as a PCR template in the nested PCR. The detailed primer combinations for the primary and the nested PCRs are listed in Table 1. Cycling conditions were the same as described in corresponding references for the PCR primers (Table 1). All PCRs were performed for 35 cycles in a 50-μl reaction mixture containing 10 μl of 5× Mg^{2+}-free GoTaq PCR buffer (Promega), 1.25 mM $MgCl_2$, 15 pmol of each primer, 200 μM of each deoxynucleoside triphosphate, ~10 ng extracted total sponge DNA or 2 μl of diluted PCR product, and 2.5 U GoTaq DNA polymerase (Promega). PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under UV transillumination. PCR products from three separate amplification reactions were precipitated using ethanol to clean the product in addition to increasing the DNA concentration.

DGGE analysis was performed using a model DGGE-2001 electrophoresis system (C.B.S. Scientific Company Inc., CA) with a denaturing gradient of 30 to 70% in a 7.5% polyacrylamide gel, following the manufacturer's instructions. The gel was made from 12 ml of 0% denaturing solution (2 ml of 50× Tris-acetate-EDTA, 18.8 ml of 40% acrylamide–bis-acrylamide [37.5:1], and 79.2 ml of water) and 12 ml of 100% denaturing solution (2 ml of 50× Tris-acetate-EDTA, 18.8 ml of 40% polyacrylamide solution, 79.2 ml of formamide, and 42 g of urea) using a GM-40 gradient maker according to the manufacturer's instructions. Prior to casting, 80 μl of ammonium persulfate and 6 μl of TEMED were added to each solution. PCR products were mixed with a one-third volume of 10× sucrose loading buffer, and DNA fragments were separated for 16 h at 100 V and 60°C. The gel was stained for 20 min with ethidium bromide and documented using a UV transillumination and VisiDoc-It imaging systems (UVP).

Individual bands were stabbed with pipette tips, and each stab was placed into 0.5-ml microcentrifuge tubes with 20 μl of sterile water. The tubes were vortexed vigorously and incubated overnight at 4°C. PCR amplifications were carried out using 1 μl of the DNA eluted from the bands and the corresponding regular (non-GC-clamped) primers of these bands for 30 cycles as described previously. Again, PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under UV transillumination. The correctly sized products were cloned into a pGEM-T Easy vector (Promega) prior to being transformed into *E. coli* cells. Clones derived from individual bands were treated as a mini clone library. The plasmids carrying inserts of the correct size were sequenced using the T7 or SP6 primers.

Sequence and phylogenetic analysis. PCR products and plasmids were sequenced at the University of Hawaii DNA Core Sequencing Facility on an Applied Biosystems 3730 automated DNA sequencer. Sequences were edited with Chromas Lite, version 2 (Technelysium). Sequence identifications were determined using the BLAST (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov). All clone sequences with ≥99% sequence identity were assigned into one group using the FastGroup II program (http://biome.sdsu

.edu/fastgroup/) and were considered to represent the same species, based on the definitions used in other studies of ITS rRNA in fungi (44, 46, 51, 68). Each of the fungal ITS or 18S rRNA gene sequences from each species and the matched sequences from GenBank were aligned with ClustalX (80). The aligned sequences were imported into PAUP* 4.0b10 (78). Neighbor-joining trees were estimated based on pairwise genetic distances on the basis of all substitutions with the Jukes-Cantor distance parameter. The quality of the branching patterns for neighbor joining was assessed by bootstrap resampling of the data sets with 1,000 replications.

Nucleotide sequence accession numbers. Sequences obtained in this study are available in GenBank under accession numbers EU084999 and EU085000, EU085008 to EU085015, EU085019 to EU085029, and EU915304 to EU915354.

RESULTS

Feasibility of using existing fungal primers for the assessment of uncultured fungal communities in marine sponges. To reveal fungal communities in marine sponges, eight pairs of primers (ITS1-F/ITS4-B, ITS3/ITS4, NS1/NS8, ITS1F/ITS4, FR1/EF390, ITS1/ITS4, nu-ssu-0817/nu-ssu-1196, and ITS5/ITS4B) were used to amplify fungal rRNA genes or ITS regions from the marine sponge *Suberites zeteki*. Only PCR amplifications using the latter four primer pairs yielded products of the expected sizes, and their products were used to construct four fungal rRNA gene or ITS clone libraries. Only a small fraction (n = 5, 4%) of the sampled clones (n = 139) carried fungal inserts; their sequences matched two uncultured fungus clones. Therefore, these primers are not suitable for direct assessment of fungal diversity in sponges.

In addition, 11 pairs of primers (Table 1), which had been successfully used to amplify diverse fungal taxa from different natural habitats in DGGE analyses (4, 11, 21, 25, 43, 56, 75), were used to amplify fungal communities from the total genomic DNA of the marine sponge *S. zeteki* in conventional PCRs or nested PCRs. With the exception of primer set 4 and 5, all PCRs yielded products of the expected sizes, and PCR products were separated using DGGE (data not shown). To determine which bands represent fungal taxa, the major bands were cloned and sequenced. The DGGE bands and fungal taxa resulting from individual primer sets are summarized in Table 1. Several attempts to clone PCR products of DGGE bands resulting from primer set 3 failed to obtain inserts of correct size. None of the clones derived from primer sets 1 and 2 contained fungal inserts. Products of these three primer sets were excluded from further analysis. All of the clones derived from DGGE bands of primer set 7 carried fungal inserts. Clones that resulted from bands of primer sets 8 and 10 contained inserts from both fungi and sponges. The percentage (n = 16, 94%) of fungal inserts in clones derived from the primer set 8 was much higher than that of the inserts derived from the primer set 10 (n = 46, 75%). Nevertheless, clones derived from primer set 10 yielded more fungal taxa (23 species) than those of primer sets 7 (7 species) or 8 (4 species). Overall, primer set 7 had the highest specificity for fungal target when used in conventional PCR, but application of primer set 10 in the nested PCRs yielded the most diverse fungal taxa.

DGGE analysis of fungal communities in marine sponges. DGGE analysis of PCR products amplified from three individuals of the marine sponges *M. armata* and *S. zeteki* using primer set 10 indicated that the band pattern of fungal communities differed in these two sponge species (Fig. 1). Band patterns of fungal communities in these two sponges were also different from those in seawater. Band patterns of fungal communities differed slightly among three individuals of *M. armata* and had little variation among three individuals of *S. zeteki*. In addition, DGGE analyses of PCR products derived from the sponges and seawater using primer sets 7 and 8 were consistent with the above results (data not shown). Band A was present in *M. armata*, and band B was only found in the sponge *S. zeteki* and seawater. Therefore, DGGE analyses suggested that two sponges may harbor largely different fungal communities, but potentially contain similar fungal groups as well.

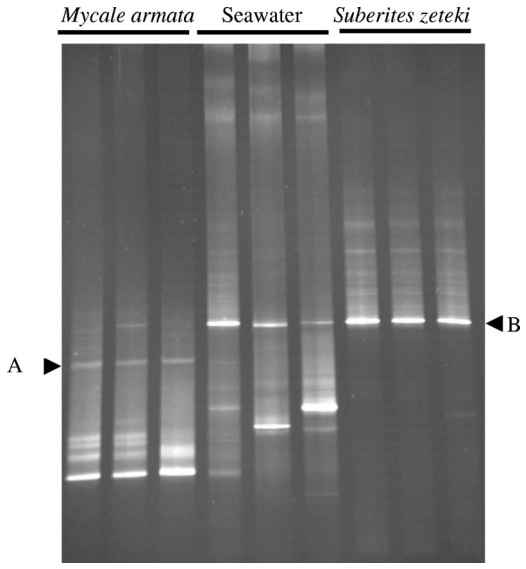

FIG. 1. DGGE analysis of uncultured fungi derived from marine sponges (*Suberites zeteki* and *Mycale armata*) and seawater. Each lane was loaded with PCR products amplified from individual samples using primer set 10.

Sequence analysis of fungal communities in marine sponges. Three PCRs were carried out for each of three types of samples (seawater, *Mycale armata*, and *Suberites zeteki*) using primer set 10. Products resulting from the three reactions of each sample type were individually pooled and separated using DGGE gel. To identify fungal communities in marine sponges, DGGE bands derived from seawater and the marine sponges *M. armata* and *S. zeteki* were cloned and sequenced. Of 61 positive clones from *S. zeteki* library, 46 clones contained fungal inserts, being assigned to 23 species (Table 2; Fig. 2A to C). These unique fungal sequences were affiliated with eight known taxonomic orders, with four from each of two phyla, *Ascomycota* (n = 35; 46%) and *Basidiomycota* (n = 41; 54%). Sequences of seven species from *S. zeteki* had sequence similarity of less than 99% with their reference sequences in GenBank and are potentially new fungal species (Table 2). In total, 51 clones were sampled and sequenced from *M. armata* clone libraries. Of 32 clones carrying fungal inserts, 21 species were identified. Their sequences were affiliated with five known taxonomic orders (Table 2), with four from the phylum *Ascomycota* and one from *Basidiomycota*. Six fungal species had matches with ≤99% sequence identity with their closest relatives in GenBank and are potentially new fungal species. Clearly, sponges harbor diverse uncultured fungal communi-

TABLE 2. Taxonomic distribution of ITS clone sequences derived from seawater and the marine sponges *Suberites zeteki* and *Mycale armata*

Species ID[a]	Taxon		Closest relative (accession no.)	% Identity	No. of clones in libraries
	Phylum	Order			
SZ10.2.1	*Ascomycota*	*Capnodiales*	*Cladosporium oxysporum* (DQ912837)	99	1
SZ10.2.11		*Capnodiales*	*Cladosporium* sp. strain 7306 (EF120415)	99	1
MA10.2.12		*Capnodiales*	*Cladosporium* sp. strain 7306 (EF120415)	99	1
SW10.4.09		*Capnodiales*	*Cladosporium* sp. strain 7306 (EF120415)	99	4
MA10.2.09		*Capnodiales*	*C. oxysporum* (DQ875018)	98	2
MA10.3.08		*Capnodiales*	*C. oxysporum* (DQ875018)	99	2
SW10.2.12		*Capnodiales*	*C. oxysporum* (DQ875018)	98	1
SW10.1.09		*Capnodiales*	*C. oxysporum* (DQ875018)	98	1
SW10.5.07		*Capnodiales*	*C.* sp. HKA30 (DQ092512)	99	1
MA10.3.06		*Dothideales*	*Hortaea werneckii* ATCC 36317 (DQ168665)	100	2
SW10.3.4		*Dothideales*	*Hortaea werneckii* ATCC 36317 (DQ168665)	99	1
SZ10.3.5		*Incertae sedis*	*Aureobasidium pullulans* CBS110374 (AY139394)	98	2
SW10.5.4		*Incertae sedis*	*Aureobasidium pullulans* CBS110374 (AY139394)	98	1
SZ10.1.2		*Eurotiales*	*Penicillium brevicompactum* (DQ123641)	99	1
SZ10.1.3		*Eurotiales*	*Penicillium restrictum* NRRL 25744 (AF033459)	99	1
SZ10.1.4		*Eurotiales*	*Penicillium restrictum* FRR 332 (AY373928)	100	1
SZ10.2.12		*Eurotiales*	*Aspergillus restrictus* ATCC 16912 (AY373864)	99	1
SZ10.4.11		*Eurotiales*	*Penicillium oxalicum* (DQ681323)	99	1
SZ10.5.8		*Eurotiales*	*Penicillium steckii* NRRL 35367 (DQ123665)	100	3
MA10.1.5		*Eurotiales*	*Aspergillus flavus* FG38 (EU030347)	99	1
MA10.2.13		*Eurotiales*	*Aspergillus flavus* FG38 (EU030347)	98	2
SW10.3.06		*Eurotiales*	*Aspergillus flavus* FG38 (EU030347)	100	1
SZ10.2.6		*Hypocreales*	*Hypocreales* sp. strain LM9 (EF060402)	99	1
SZ10.5.13		*Hypocreales*	*Hypocreales* sp. strain LM9 (EF060402)	98	1
SW10.5.1		*Hypocreales*	*Gibberella moniliformis* NRRL 43697 (EF453174)	99	1
SW10.4.12		*Saccharomycetales*	*Candida tropicalis* isolate16 (EF216862)	100	1
MA10.3.10		*Pleosporales*	Uncultured fungus clone 24b (EU003082)	99	1
SW10.4.4		*Pleosporales*	Uncultured fungus clone 24b (EU003082)	99	1
SW10.4.5		*Pleosporales*	Uncultured fungus clone 24b (EU003082)	99	1
MA10.2.15		*Pleosporales*	*Ascomycota* sp. strain LM107 (EF060476)	99	1
SW10.4.08		*Pleosporales*	*Phoma putaminum* isolate P-14 (AM691009)	98	1
SZ10.7.4	*Basidiomycota*	*Agaricales*	*Schizophyllum commune* HNO34 (AF280758)	99	1
SZ10.2.2		*Corticiales*	*Phlebia* sp. strain olrim964 (AY787680)	86	1
SZ10.2.9		*Malasseziales*	*Malassezia restricta* CBS 7877 (AY743636)	98	3
SZ10.2.13		*Malasseziales*	*Malassezia restricta* CBS 7877 (AY743636)	98	2
SZ10.5.3		*Malasseziales*	*Malassezia restricta* CBS 7877 (AY743636)	98	1
SZ10.5.5		*Malasseziales*	*Malassezia restricta* CBS 7877 (AY743636)	99	2
MA10.3.17		*Malasseziales*	*Malassezia restricta* CBS 7877 (AY743636)	99	2
SZ10.3.12		*Malasseziales*	*Malassezia* clone gbCTA7_004 (DQ900961)	99	1
SZ10.4.4		*Malasseziales*	*Malassezia* clone gbCTA7_004 (DQ900961)	98	2
SZ10.4.5		*Malasseziales*	*Malassezia* clone gbCTA7_004 (DQ900961)	99	1
SW10.1.1		*Malasseziales*	*Malassezia* clone gbCTA7_004 (DQ900961)	98	7
SW10.1.5		*Malasseziales*	*Malassezia* clone gbCTA7_004 (DQ900961)	98	1
MA10.2.08		*Malasseziales*	*Malassezia* clone gbCTA7_004 (DQ900961)	99	1
SZ10.5.6		*Malasseziales*	Fungus clone (AM260792)	99	1
MA10.2.5		*Malasseziales*	Fungus clone (AM260792)	99	3
SZ10.1.5		*Malasseziales*	*Malassezia restricta* FRR 332 (AJ437695)	99	13
MA10.3.4		*Malasseziales*	*Malassezia restricta* FRR 332 (AJ437695)	99	2
MA10.3.5		*Malasseziales*	*Malassezia restricta* FRR 332 (AJ437695)	99	1
MA10.3.11		*Malasseziales*	*Malassezia restricta* FRR 332 (AJ437695)	100	1
MA10.3.16		*Malasseziales*	*Malassezia restricta* FRR 332 (AJ437695)	99	4
SW10.3.5		*Malasseziales*	*Malassezia restricta* FRR 332 (AJ437695)	99	1
SW10.2.9		*Malasseziales*	*Malassezia restricta* FRR 332 (AJ437695)	100	1
SW10.4.07		*Malasseziales*	*Malassezia restricta* FRR 332 (AJ437695)	99	2
MA10.3.1		*Malasseziales*	*Malassezia globosa* CBC1 (AY387136)	99	1
MA10.3.3		*Malasseziales*	*Malassezia sympodialis* CBS 8740 (EF140668)	87	1
MA10.2.06		*Malasseziales*	*Malassezia sympodialis* WF42 (AY387181)	85	1
SW10.5.3		*Malasseziales*	*Malassezia sympodialis* WF42 (AY387181)	85	3
MA10.1.11		*Malasseziales*	*Malassezia sympodialis* MA 477 (AY743639)	85	1
MA10.3.12		*Malasseziales*	*Malassezia sympodialis* MA 477 (AY743639)	85	1
SW10.2.5		*Malasseziales*	*Malassezia sympodialis* MA 477 (AY743639)	85	2
SW10.2.08		*Malasseziales*	*Malassezia sympodialis* MA 477 (AY743639)	85	1
SW10.5.09		*Malasseziales*	*Malassezia sympodialis* MA 477 (AY743639)	85	1
MA10.1.12		*Malasseziales*	Uncultured *Malasseziales* clone BD19 (DQ317381)	99	1
SW10.3.13		*Malasseziales*	Uncultured AMF fungus (AY267224)	99	1
SW10.5.2		*Malasseziales*	*Malassezia globosa* CBS 7966 (AY743630)	99	1
SW10.5.06		*Malasseziales*	*Malassezia globosa* CBS 7966 (AY743630)	97	1
SW10.5.08		*Malasseziales*	*Malassezia globosa* CBS 7966 (AY743630)	98	1
SW10.5.11		*Malasseziales*	*Malassezia globosa* CBS 7966 (AY743630)	98	1
SW10.2.10		*Malasseziales*	*Malassezia sympodialis* MA 424 (AY743654)	83	1
SZ10.7.2		*Polyporales*	*Basidiomycete* sp. strain LC5 (AY605709)	99	4

[a] The species ID number indicates the sample source (SZ, *Suberites zeteki*; MA, *Mycale armata*; SW, seawater) and clone number.

ties, but their composition differs in two sponge species, *S. zeteki* and *M. armata* (Table 2; Fig. 2A to C).

Members of *Malasseziales* (*Basidiomycota*) were dominant sequences in sponge and seawater clone libraries, ranging from 58 to 64% (Table 2). The compositions of fungi in *M. armata* and seawater were very similar, but the order of *Saccharomycetales* was identified in seawater, not in *M. armata*. Finally, members of three orders (*Polyporales*, *Agaricales*, and *Corticiales*) were only present in *S. zeteki*.

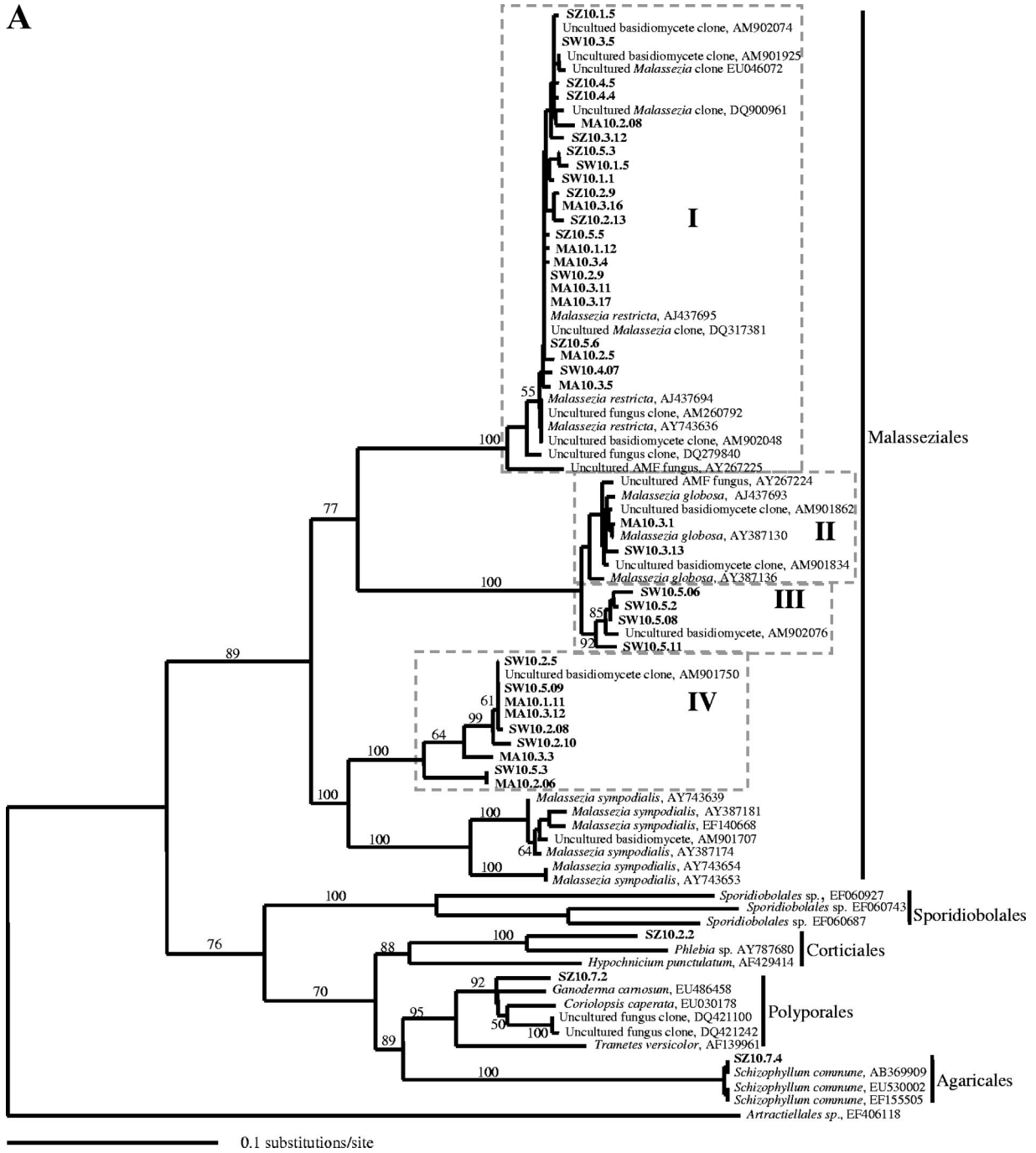

FIG. 2. Neighbor-joining phylogenetic trees (*Basidiomycota* [A] and *Ascomycota* [B and C]), based on rRNA-ITS sequences of fungal sequences derived from marine sponges and seawater. Numbers above or below branches indicate bootstrap values (>50%) from 1,000 replicates. Uncultured fungal sequences from marine sponges and seawater are in bold, while cultured sequences, which are from references 40 and 71, are in a regular font. Sequences derived from sponges are labeled with the sponge name, followed by the clone ID and isolate ID for uncultured fungi and cultured fungi, respectively. Marine fungal clades are boxed with a solid line (B and C). Four basidiomycete clades are boxed with a dashed line (A). MA, *Mycale armata*; SZ, *Suberites zeteki*; GF, *Gelliodes fibrosa*; HC, *Haliclona caerulea*.

Phylogenetic analysis of uncultured fungi derived from marine sponges. Phylogenetic analyses indicated that fungal sequences derived from marine sponges and seawater were closely affiliated with those derived from diverse habitats, including marine environments, in GenBank (Fig. 2A to C). Basidiomycete ITS sequences clustered into four groups, corresponding to four taxonomic orders (*Corticales*, *Polyporales*, *Agaricales*, and *Malasseziales*) (Fig. 2A). The vast majority (n = 37) of the identified basidiomycete sequences belonged to *Malasseziales*, clustered into 4 clades. Members of clade I, including twenty-two sequences derived from both marine sponges (n = 17) and seawater (n = 4), were closely affiliated

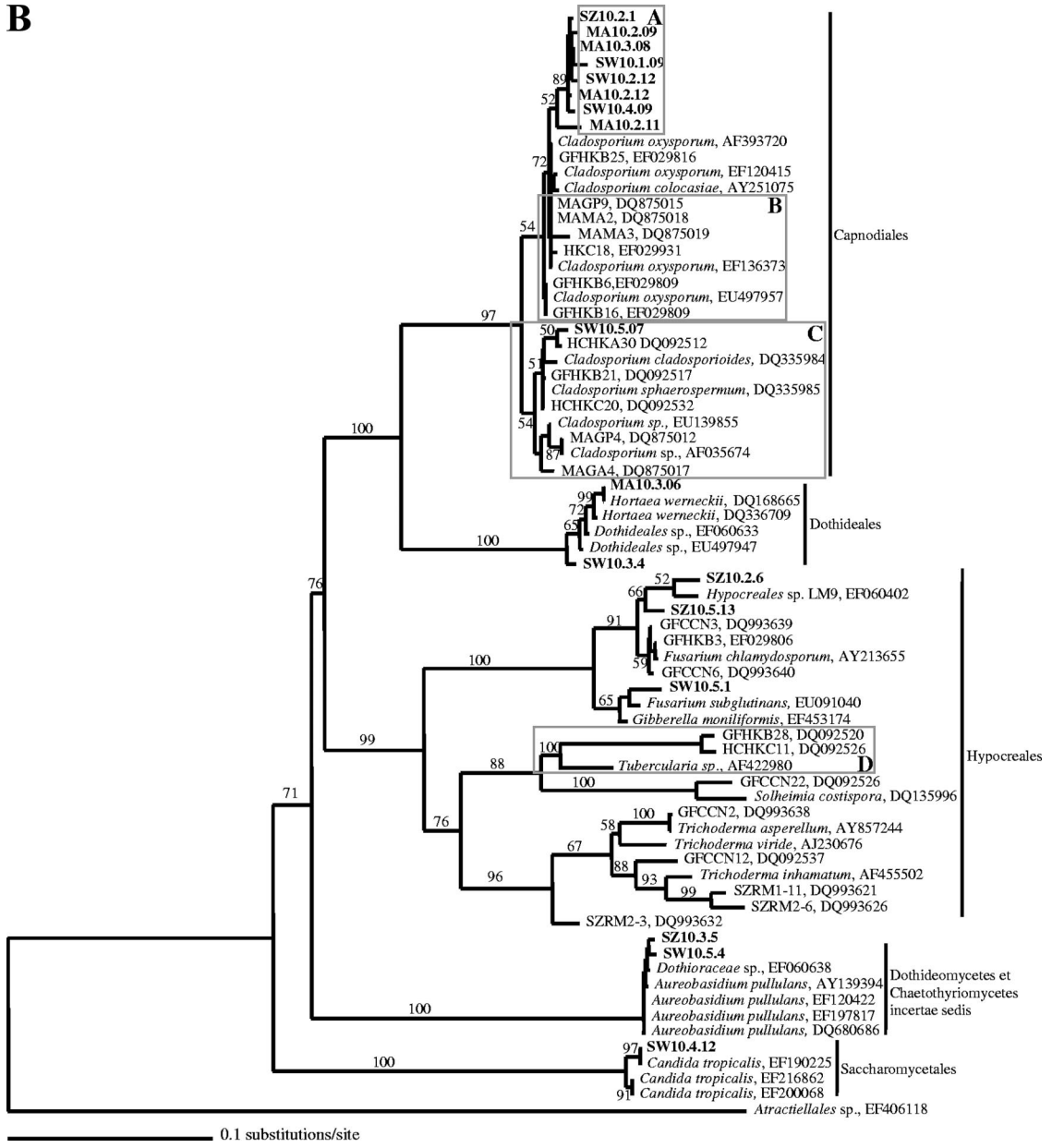

Fig. 2—*Continued.*

with *Malassezia* sequences, which included *Malassezia restricta* sequences derived from soil nematodes in Central European forests (70) and dogs with otitis externa (13, 18) and clone sequences derived from peat (5), house dust (63), dry valley and mountain soils in the Ross Sea region of Antarctica (4), the ectomycorrhizal root tip of *Pinus sylvestris* (EU046072), as well as deep-sea sediments (45). Two members of clade II derived from *M. armata* and seawater were clustered with a sequence of *M. globosa* from soil nematodes (70), elephants and humans (29), as well as other sequences of uncultured basidiomycete clones derived from soils from northern Sinaloa (Mexico) (55) and house dust (63). Four sequences from seawater along with one uncultured basidiomycete sequence derived from house dust (63) formed clade III. Members of clade IV included sequences derived from seawater and *M. armata* and one uncultured basidiomycete sequence derived from house dust (63). Three other sequences (SZ10.2.2, SZ10.7.2, and SZ10.7.4) clustered individually into three groups (*Corticiales*, *Polyporales*, and *Agaricales*, respectively). Because of the low sequence similarity (86%) with *Phlebia* sp., sequence SZ10.2.2 is potentially a new fungal species.

Thirty-one ascomycete sequences derived from marine sponges and seawater clustered into seven groups, corresponding to seven taxonomic orders (*Dothideomycetes et Chaetothyriomycetes incertae sedis*, *Capnodiales*, *Dothideales*, *Hypocreales*, *Eurotiales*, *Pleosporales*, and *Saccharomycetales*) (Fig. 2B and C). Along with culturable fungal sequences from sponges and other marine environments, nine of these sequences were

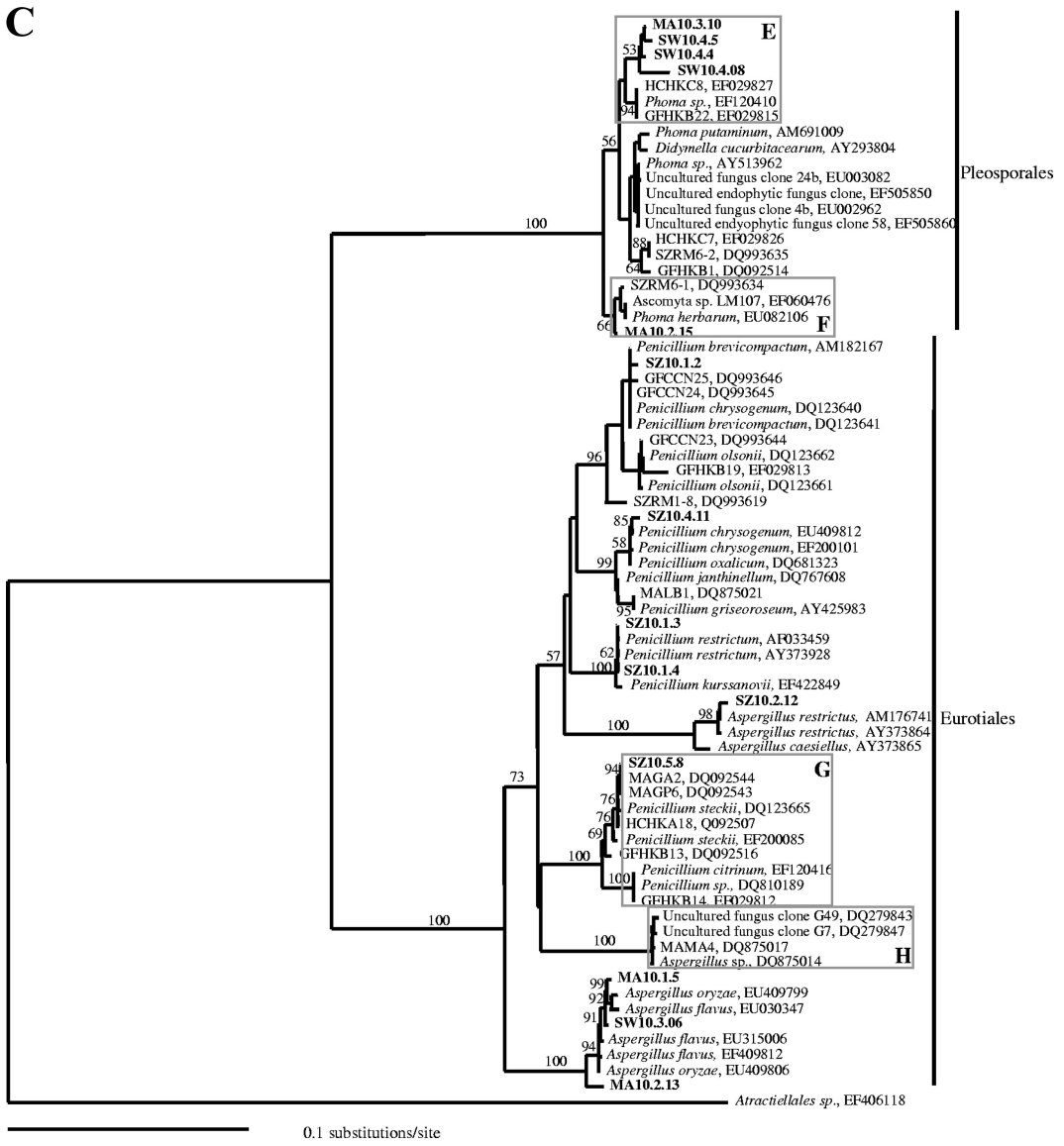

Fig. 2—*Continued.*

clustered into *Capnodiales*. Eight (SZ10.2.1, SZ10.2.11, MA10.2.12, MA10.3.08, MA10.2.09, SW10.2.12, SW10.1.09, and SW10.4.09) of these nine uncultured fungal sequences formed marine clade A, which did not include any reference sequences in GenBank as their phylogenetic neighbor. It is likely that these 8 sequences belong to a new phylotype group. Sequence SW10.5.7 was clustered into marine clade B with sequences of *Cladosporium* spp. derived from sponges, marine animals, and seawater. In addition, several culturable fungal sequences derived from marine sponges formed into marine clade C along with those derived from corals and deep sea sediments. Members of the *Dothideales* included MA10.3.06 and SW10.3.4 and uncultured reference sequences from the Pacific Ocean and hypersaline environments. Three sequences (SZ10.2.6, SZ10.5.13, and SW10.5.1) were members of *Hypocreales*. The two sequences from *S. zeteki* were closely related to *Hypocreales* sp. from the Pacific Ocean, forming marine clade D. Two sequences (SZ10.3.5 and SW10.5.4) were closely related to *Dothioraceae* sp. from the Pacific gyre and to the ubiquitous saprophyte *Aureobasidium pullulans*, classified as *Dothideomycetes et Chaetothyriomycetes incertae sedis* according to Eriksson et al. (22). Finally, one sequence (SW10.4.12) was clustered into *Saccharomycetales* with three other yeasts, corresponding to marine clade E.

Eurotiales included nine sequences from marine sponges and seawater (Fig. 2C). These sequences were closely affiliated with sequences of cultured *Penicillium* spp. and *Aspergillus* spp. derived from Hawaiian marine sponges, seawater, and terrestrial plants as well as uncultured ones from seawater and deep sea sediments. The *Eurotiales* included two marine clades (G and H). Members of the *Pleosporales* group only contained sequences from *M. armata* and seawater. Four of these sequences (MA10.3.10, SW10.4.5, SW10.4.4, and SW10.4.08) were clustered with two fungal isolates from Hawaiian marine

sponges and one *Phoma* sp. isolated from the diseased Great Barrier Reef corals. The other sequence (MA10.2.15) formed a different cluster with one sponge isolate, one fungal clone from Pacific Gyre, and *P. herbarum* isolated from the Great Barrier Reef corals. Finally, two marine clades (E and F) were identified in the *Pleosporales*.

DISCUSSION

The ecological importance of fungi in terrestrial environments (e.g., soil) has been very well documented (2). However, the ecological roles of fungi in marine environments are often underestimated or ignored completely (35, 40, 59). As a rich reservoir for diverse microbial groups, marine sponges have been shown by cultivation-dependent methods to harbor diverse groups of filamentous fungi and yeast (26, 49, 84). In this study, for the first time, we applied molecular techniques to investigate fungal communities in the marine sponges *Suberites zeteki* and *Mycale armata* and reported the feasibility of using existing fungal primers for the assessment of fungal communities in marine sponges. Our results indicated that the two sponge species harbor diverse fungal communities, including representatives of two phyla (*Basidiomycota* and *Ascomycota*) and of 11 taxonomic orders. Five of these taxonomic orders (*Malasseziales*, *Corticiales*, *Polyporales*, *Agaricales*, and *Dothideomycetes et Chaetothyriomycetes incertae sedi*s) are identified for the first time in marine sponges.

PCR primers play a crucial role in the molecular assessment of environmental microbes. The specificity of the primer pairs is vital in this context to allow selective or enriching amplifications of fungal rRNA genes from environmental DNA (59). Amplification of fungal rRNA-ITS regions from the total genomic DNA of *S. zeteki* using four pairs of fungal primers (ITS1-F/ITS4-B, ITS3/ITS4, NS1/NS8, and ITS1F/ITS4) did not yield any PCR product. PCRs using the universal primers ITS1/ITS4 (88) and several other primers with the ability to preferentially amplify fungal rRNA genes from environmental samples (FR1/EF390, nu-ssu-0817/nu-ssu-1196, and ITS5/ITS4B) (9, 24, 54, 82) yielded mostly sponge rRNA genes. Several factors may contribute to the failures of these amplifications. First of all, fungal primers used in the construction of clone libraries may have a low specificity to their fungal targets in the presence of many copies of nuclear ribosomal genes of *S. zekeki*. Phylogenetically, sponges and fungi are closely related (8, 58, 61, 73); many fungal primers, designed to target fungal 18S rRNA gene or ITS regions, have been observed to match with rRNA gene sequences of Porifera and other organisms (59). The absence or presence of fungal rRNA products derived from primer sets 1, 2, and 7 clearly indicates the different fungal specificity of these primers. Secondly, the abundance of fungi can be much lower than that of bacteria in marine sponges. A typical milliliter of seawater contains 10^3 fungal cells and 10^6 bacteria (71). Without the specific selection during the filter-feeding process, fungal cells in sponges could be several orders of magnitude lower than those of bacterial cells. Finally, the properties of fungal cell wall materials could bias the DNA extraction protocol against fungi and thus produce a low yield of fungal genomic DNA. Identification of diverse fungal taxa from the nested PCR products seems to support the last two points (Table 1).

The nested PCR strategy has been used to detect fungal communities in several terrestrial environments (4, 43). Three DGGE primer sets (7, 8, and 10) were successful in the amplification of fungal rRNA genes or ITS regions from *S. zeteki* (Table 1). However, all sequences derived from primer sets 7 and 8 belonged to *Agaricales* (*Basidiomycota*) and matched only to one fungal clone (WIM108, AM114819), which was derived from soil with the sequence identity ranging 98 to 100% (data not shown). Although these two primer sets have very high fungal specificity, their feasibility for assessing fungal communities in marine sponges is still questionable because they can only detect a very few fungal taxa. On the other hand, PCR amplification using primer set 10 yielded many more diverse fungal taxa (Table 1) than that using primer sets 7 and 8. The target of primer set 10 is the 450-bp ITS regions that are quite divergent and differ between species within a genus. Those regions have commonly been used to delineate lower taxonomic ranks, such as genera and species (12, 88). Furthermore, ITS4 and ITS3 are designed to amplify a broad range of fungi (88), while ITS1F is specific to fungi (24). Therefore, the nested PCR protocol using primer set 10 can provide a satisfactory degree of success in discriminating against the sponge DNA while maintaining a broad range of fungal compatibility (Table 1; Fig. 2A to C).

Fungi can be easily isolated from marine sponges. As of now, application of the morphology-based approach has identified less than 200 fungal isolates in marine sponges (38, 57, 64). Many of those isolates belong to the genera commonly found in terrestrial habitats (e.g., *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Trichoderma*, and *Penicillium*). The diversity of culturable fungal assemblages varied greatly, ranging from 0 to 21 fungal genera per sponge species (42, 64). Fourteen (*Capnodiales*, *Eurotiales*, and *Dothideales*) and 20 (*Pleosporales*, *Hypocreales*, *Phyllachorales*, *Eurotiales*, and *Diaporthales*) fungal isolates were cultured from *M. armata* and *S. zeteki*, respectively (49, 85). Efforts of this study added two (*Pleosporales* and *Malasseziales*) and six (*Dothideomycetes et Chaetothyriomycetes incertae sedis*, *Malasseziales*, *Polyporales*, *Capnodiales*, *Agricales*, and *Dothideales*) additional fungal orders to the inventory of fungi in the marine sponges *M. armata* and *S. zeteki*, respectively. However, members of the two orders *Phyllachorales* and *Diaporthales*, which were identified in cultured fungal populations, were not present in uncultured fungal sequence from *S. zeteki*. Compared with culture-dependent approaches, DGGE and library construction revealed much more diverse fungal groups in the marine sponges *M. armata* and *S. zeteki* (Fig. 2A to C) (49, 85). This discrepancy between cultivation-dependent and cultivation-independent approaches has been commonly observed in the ecological studies of bacterial communities in marine sponges and many other natural habitats (34).

The nature and ecological function of sponge-inhabiting fungi remain elusive. Sponge-inhabiting fungi have been proposed to belong to two categories: "resident fungi" and "transient fungi" (49). The "resident fungi" are sponge specific, while the "transient fungi" are those fungi resulting from "wash-in spores" trapped in sponges during the filter feeding process. Several lines of evidence appear to support a symbiotic association between sponges and fungi. Compelling evidence for symbiosis of a yeast with sponges of the genus *Chon-*

drilla has been observed in extensive studies of both adult sponge tissue and reproductive structures, indicating the vertical transmission of the yeast symbionts (52). The antifungal response of the sponge *S. domuncuta* at molecular levels indicates an intriguing relationship between sponges and fungi (61). In addition, the horizontal gene transfer of a mitochondrial intron from a fungus to sponges suggested a "true symbiotic" relationship between fungi and sponges (73). Although several phylotypes were exclusively found in *S. zeteki*, no inclusive evidence from this study supports the existence of "resident fungi" (Fig. 2A to C). Most fungal phylotypes are found in both marine sponges and seawater. Furthermore, seawater and *M. armata* harbor similar, but not identical, fungal communities. However, the true diversity and composition of marine fungi in these two marine sponges need to be further analyzed in the future for several reasons. First, selection of DGGE bands for cloning and sequencing analyses is not totally random and inclusive. Some fungal taxa with low or medium abundance in a DGGE gel may be completely missed. Second, it is well established that a bias may occur in PCR amplification from mixture of rRNA templates. For example, Polz and Cavanaugh (65) found that a template containing GC-rich permutations in priming sites may be overrepresented. Particularly, the nested PCR protocol may result in the overrepresentation of certain taxa. Finally, selective amplification of certain fungal taxa from sponge total genomic DNA (e.g., primer sets 7 and 8 in this study) clearly indicated the limitation of the existing fungal primers. Therefore, selection of fungal primers with the ability to preferentially amplify against sponge rRNA-ITS regions but to also target large groups of fungal taxa is clearly a challenge in the study of sponge-associated fungal community.

Currently, rather than a taxonomic group, "marine" fungi are considered only as an ecologically or physiologically defined microbial group (85). The fact that many sponge-inhabiting fungi have the ability to produce novel compounds that are absent in the cultures of their terrestrial counterparts clearly indicates the novel metabolic or genetic capacity of these fungi with sponge origin (41, 42, 66, 84). However, phylogenetic analysis of sequences derived from DGGE bands did not reveal any "sponge-specific" fungal groups, but indeed suggested the existence of "marine fungus" phylotypes (Fig. 2A to C). Further large-scale phylogenetic analysis of unculturable and culturable fungi from both sponges and other marine environments (e.g., sediments and seawater) may provide better insights in this regard. On the whole, the results of this report and other studies suggest that "marine" fungi may exist in sponges. The close relationship of some fungal sequences derived from marine sponges with those from other marine habitats points to the presence of cosmopolitan "marine fungi" in the ocean. The identified marine fungi only account for about 5% of total marine fungi (i.e., about 10,000 species) in the oceans. Without doubt, more studies using molecular approaches for more inclusive information are needed to investigate fungal communities in sponges. Such effort will contribute significantly to the understanding of the evolutionary relationship of sponges with fungi, of global fungal diversity, and of the biotechnology of sponge symbionts.

Surprisingly, diverse *Malassezia* phylotypes were identified in the marine sponges *M. armata* and *S. zeteki* (Table 2; Fig. 2A). *Malassezia* species are lipophilic yeasts that are members of the normal mycoflora of the human skin and are also found on the skin of a variety of animal species (13). They are associated with a variety of dermatological disorders of human skin, including but not limited to, atopic dermatitis, dandruff, and folliculitis, and they are also implicated in several skin disorders in animals, mainly otitis externa and dermatitis (13, 19, 27, 28, 53). *Malassezia* species have been isolated from humans, animals, soils, house dust, and deep sea sediments (4–6, 13, 29, 45, 60, 63). This study is the first report of *Malassezia* spp. in marine sponges and invertebrates. To the best of our knowledge, their species diversity in sponges is the greatest diversity of *Malassezia* spp. from a single host reported so far (Fig. 2A). Therefore, our findings indicate that sponges may represent an ecologically important hot spot. Future study of this fungal group using culture-dependent and -independent approaches should reveal useful information on the ecological significance of *Malassezia* spp. in marine sponges and other invertebrates.

In conclusion, diverse unculturable fungi have been identified in the marine sponges *S. zeteki* and *M. armata*. A small fraction of these fungi belongs to fungal genera of the culturable fungi; the majority of them are members of fungal taxa identified in marine sponges for the first time. In addition, the nested PCR protocol using the ITS primers (Table 1) provides a useful vehicle to explore the unculturable fungi in sponges and other marine invertebrates. Several "marine" fungal clades identified in these two marine sponges support the existence of "marine fungal phylotypes." Marine sponges, as a specialized marine habitat, contain previously undescribed taxa of sponge-inhabiting fungi, some of which may be new species. This is the first report of unculturable fungi in marine sponges, and it brings new insights into the ecology of sponge-associated microbial communities.

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